FURTHER MOLECULAR AND CATALYTIC CHARACTERIZATION OF UTERINE PHOSPHORYLASE *b*

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1. Introduction

Since 1958 the glycogen phosphorylase activity from mammalian uteri has been the subject of several reports [1–3]. The procedures for isolation of the homogenous enzyme were developed later [4–6]. Uterine enzyme was characterized as a form different from both liver and skeletal muscle phosphorylases [2,4,5]. Here we describe an advanced procedure for purification of the cow uterine enzyme using AMP–Sepharose chromatography. Some of the characteristics of the homogenous uterine phosphorylase, not previously reported, are described. The results show that its molecular and catalytic properties are very similar to those of the phosphorylase isozyme I. Part of this work was reported in [7].

2. Materials and methods

Swine liver glycogen and Tris were obtained from Olaine, USSR. Glc-1-P, Glc-6-P and AMP were purchased from Reanal, Hungary; mercaptoethanol was from Serva and β-glycerophosphate from Fluka AG. AMP–Sepharose was a generous gift of Pharmacia Fine Chemicals. All other chemicals were of highest grade obtainable. ω-Aminohexyl–Sepharose 4B with 1.3 equiv./ml of ω-aminohexyl groups was prepared as in [8]. A modified Cori's assay was used to measure the phosphorylase activity [9]. The reaction mixture contained 20 mM β-glycerophosphate, 1% glycogen, 16 mM Glc-1-P and 1 mM AMP. Phosphorylase units were measured as μmol P_i/min at 30°C and pH 6.8. Protein concentration was determined either according to [10] or by direct measurement of A_{280}, using the phosphorylase index $E^{1\%}_{1\,cm}$ = 13.2 [11]. Enzyme fractions were concentrated by ultrafiltration (Diaflo cell, Amicon, with XM 100 filter). PLP was determined as in [12], using a Pye Unicam SP 8000 spectrophotometer. Analytical isoelectric focusing was done in an LKB column, model 8101.

Abbreviations: Glc-1-P, glucose-1-phosphate; Glc-6-P, glucose-6-phosphate; P_i, inorganic phosphate; PLP, pyridoxal-5′-phosphate

3. Results and discussion

Here we used the procedure described [6] for isolation of homogenous phosphorylase *b* from cow myometrium but modified at the final stage. The method used previously involved affinity adsorbtion on high-molecular-weight glycogen in a batch procedure, column hydrophobic chromatography and, finally, column chromatography on glycogen–hydrazidosuccinyl–Sepharose. Here, the immobilized glycogen was replaced by another affinity adsorbent, commercial AMP–Sepharose. The suspension (10 mg protein) was applied to a column of AMP–Sepharose (5 × 1.2 cm) equilibrated with 50 mM Tris–HCl/1 mM EDTA/0.1 mM-mercaptoethanol (pH 7). The column was then developed with the same buffer plus 10 mM Glc-6-P. The dialysed homogenous preparation of 1.2 mg phosphorylase *b* was purified ~2500-fold, as compared to the initial extract and the recovery was ~7%. The specific activity of the enzyme was 16.5 units/mg protein at 16 mM Glc-1-P. A typical elution pattern is depicted in fig.1.

AMP–Sepharose, synthesized by immobilization of periodate-oxidized AMP on hydrazidoadipinyl–Sepharose, was an ineffective adsorbent of uterine

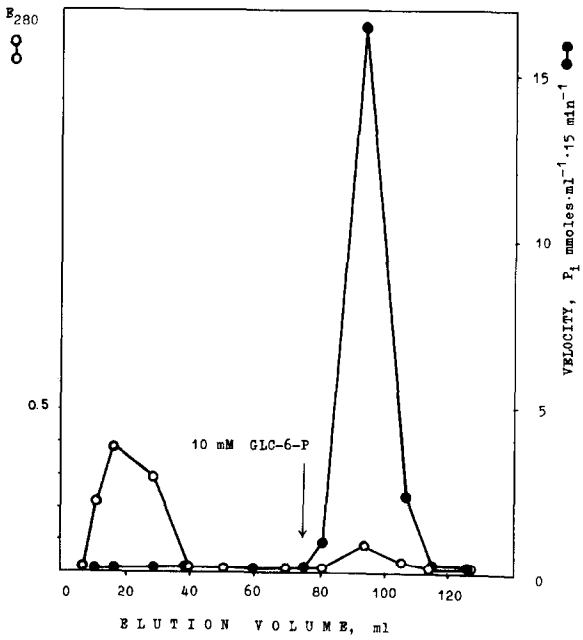

Fig.1. Elution pattern of cow uterine phosphorylase *b* from AMP–Sepharose column chromatography. For conditions see sections 2 and 3.

phosphorylase *b* (not shown). Here we used a commercial preparation of AMP–Sepharose, a product of the coupling N^6 (6-aminohexyl)-AMP to Sepharose. The isoelectric point was estimated by electrofocusing as in [8]. The pI of the main peak was at 5.7. This compares with pI values of 6.25 and 6.2 for the phosphorylase isozymes from skeletal muscle and liver, respectively [8,13]. The PLP content in uterine phosphorylase was estimated by a modified sensitive spectrophotometric method based on determination of the hydrazone in acid medium after the reaction of PLP with 4-nitrophenylhydrazine [12]. It was calculated that 200 kg phosphorylase contained of ~1.95 mol PLP. The mol. wt of the enzyme subunits was estimated by SDS gel electrophoresis to be ~100 000 (not shown).

The relation between the concentration of AMP and the reaction rate revealed a curve of Michaelis-Menten type giving a K_a of the enzyme for AMP of 7.3 μM. The reaction mixture contained 5 μg phosphorylase/ml, 75 mM Glc-1-P, 1% glycogen and AMP from 4–40 μM in 0.1 M maleate buffer (pH 6.5). The obtained K_a^{AMP} value is in contrast to the K_a^{AMP} values for phosphorylase *b* from skeletal muscle and liver, which are 60–62 μM and 75 μM, respectively [14,15]. As seen in fig.2, uterine phosphorylase *b* was inhibited by caffeine. The effect of caffeine concentration on the activity of the uterine enzyme was measured at 0.01 mM and 1 mM AMP. The former value is comparable with AMP concentration in the uterus [16], the latter is the AMP concentration employed in the routine determination of the phosphorylase *b* activity. AMP, as a caffeine analog, protects the enzyme against the action of this inhibitor.

We measured initial rates of the synthetic reaction of inactive phosphorylase at various concentrations of Glc-1-P in the presence of the following physiological inhibitors of glycogen phosphorolysis: glucose, Glc-6-P and ATP. In all cases the curves were *S*-shaped. The cooperativity coefficients, *h*, were cal-

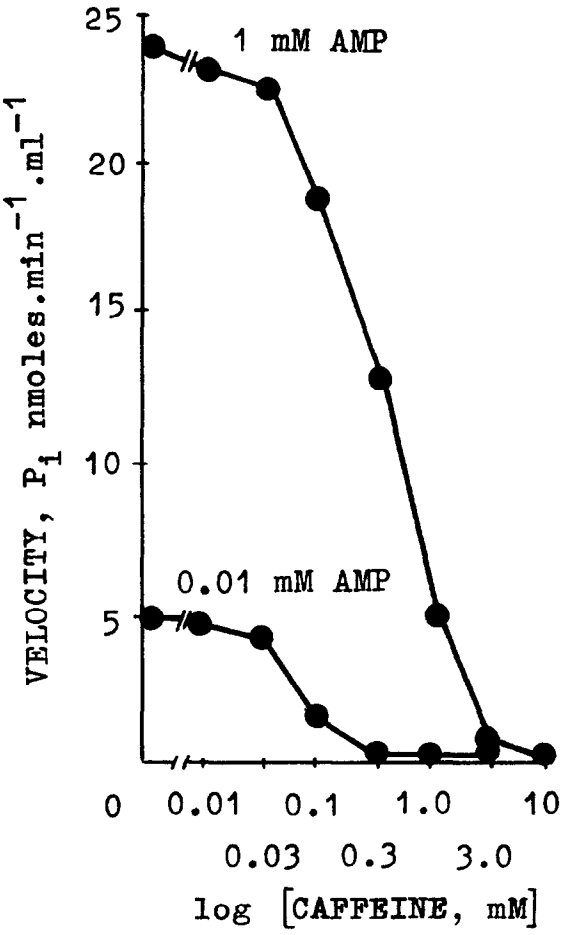

Fig.2. The inhibition of uterine phosphorylase *b* by caffeine and the protecting effect of AMP. Assayed at 30°C in 20 mM β-glycerophosphate buffer, 0.1 M NaF, 1 mM EDTA, 1% glycogen and 16 mM Glc-1-P (pH 6.3).

culated graphically by the Hill method in coordinates $\log[V/(V_{max}-V)]$ vs $\log[S]$. The values of the Hill coefficients were equal to 1.6, 1.7 and 1.9 at 15 mM Glc-6-P, 10 mM ATP and 25 mM glucose. The $S_{0.5}$ data for these effectors were 12.8 mM, 13 mM and 8.7 mM, respectively. Therefore the data obtained suggest the existence of a positive allosteric interaction of the Glc-1-P binding sites in uterine phosphorylase *b*.

These data and [2,4,5] demonstrate that uterine phosphorylase *b* is a dimer with a mol. wt ~200 000. The conversion of inactive phosphorylase *b* to the active form, phosphorylase *a*, was not accompanied by tetramerization. The sedimentation coefficent of the enzyme was calculated to be 8.4 S and pI 5.7. The protein contained 1 mol PLP/100 kg enzyme subunit. Uterine *b*-form was inactive without AMP and had high affinity for this activator with K_a^{AMP} = 7.3 μM. It was not activated by Na_2SO_4 alone, though activated by this salt in the presence of AMP. Caffeine inhibited uterine phosphorylase *b*. The smooth muscle phosphorylase *b* did not differ from both isozyme III, i.e., skeletal muscle phosphorylase, and liver isozyme L in its binding affinity for Glc-1-P, its $s_{20,w}$ value, subunit mol. wt. and PLP content. Like phosphorylases L and III, the uterine enzyme exhibited allosteric properties in the presence of some effectors. The analyzed protein was different from both isozyme L and III in respect to its pI and K_a^{AMP} values. Whereas skeletal muscle phosphorylase *b* underwent a doubling of molecular weight during conversion to the active form, the inactive uterine enzyme did not. Uterine inactive phosphorylase differs from liver enzyme in that it undergoes additional activation by Na_2SO_4 only in the presence of AMP. It also differs from liver phosphorylase in its K_i characteristics for glucose and ATP (not shown). No significant distinction is to be drawn between uterine phosphorylase and isozyme I. Homodimer I–I is one of the components of the phosphorylase isozyme patterns in mammalian heart [17]. Its pI was found to be 5.6 [13] and K_a^{AMP}, found 9 μM [14].

Insignificant variations in uterine and isozyme I constants are to be accounted for by species specific differences. However, the pI and K_a^{AMP} values for III and L isozymes mentioned above stand in marked contrast to those for isozyme I. The identity of the uterine form with the isozyme I was also supported by the data on electrophoretic mobility of the phosphorylase from the rat uterine extract and its inhibition by antisera [13].

Therefore, the forms cited in the literature as brain phosphorylase [18], heart isozyme I [17,19], fetal isozyme [20,21] and uterine phosphorylase should be considered as the same protein species.

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